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pH Stability Studies with Avian Infectious Bronchitis Virus (Coronavirus) Strains

B. S. COWEN* AND S. B. HITCHNER

Department of Avian Diseases, New York State Veterinary College, Cornell University, Ithaca, New York 14850

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A comparison of 17 infectious bronchitis virus strains, using the same test procedure and assay system, demonstrated that stability at an acid pH is a variable characteristic of the avian coronaviruses.

The literature reveals that there are differences in sensitivity to an acid pH by members of the coronavirus group (1, 2, 4-6, 8, 10, 11, 13, 15, 16, 18). It is not clear whether these inconsistencies reflect differences in methodology or virus strains. To resolve this question, numerous coronavirus strains must be tested by using a standardized procedure and the results must be compared. With these facts in mind, the present study was conducted for the primary purpose of comparing the effect of an acid pH on several infectious bronchitis virus (IBV) strains, where the test procedure and assay system were consistent throughout.

Seventeen cell culture-cloned (three times) strains (JFB isolates have been classified as Massachusetts serotypes [10; Cowen, unpublished data] of IBV were diluted 10-fold in pH 3.0- and 7.2-adjusted (0.006 M Tris buffer) media (medium 199) as described by Ketler et al. (12). After 4 h of incubation at room temperature, the residual virus content was determined. Tenfold serial dilutions (in medium 199) were each inoculated into two (plaque assay) or three (50% end point) primary chicken kidney cell cultures prepared from 2- to 3-week-old specific-pathogen-free chickens in a manner similar to that described by Churchill (3). Growth medium consisted of medium 199 containing 5% bovine fetal serum, 10% tryptose phosphate broth, 100 U of penicillin, 100 μg of dihydrostreptomycin, and 40 U of mycostatin per ml. Inoculated cultures were held under maintenance medium (growth medium without bovine fetal serum) or were overlaid with the same medium containing agar (Difco) to a final concentration of 0.8%. After 48 to 72 h of incubation (37 C in a humidified atmosphere with 5% CO_2), cultures were stained by adding 0.5 ml of 0.1% neutral red 1 to 2 h before examination for plaques or were observed (unstained) at $\times$60 (Wild M40 inverted microscope) for cytopathic effect. The number of PFU per milliliter was calculated from the dilution giving 20 to 200 plaques per culture. Infectivity end points (mean tissue culture infective dose) were calculated by the method of Reed and Muench (14). The difference in titer between preparations held at pH 3.0 and 7.2 was determined, and the virus strain was considered stable if the average difference in two or more trials was less than 10-fold (7).

Of the 17 IBV strains tested (Table 1), only the Gray strain exhibited acid stability. It is of interest to note that the 16 acid-labile (in one or more trials) strains varied considerably in their sensitivity (reductions of 10^1 to $>10^5$) to an acid pH. Some of this variability among strains was displayed, interestingly, by members of the same serotype (e.g., JFB5 and Massachusetts 41). The majority, however, exhibited titer reductions of 10- to 100-fold. The relatively greater acid lability displayed by the Massachusetts 41 and Holte strains can not be presently explained.

The variation among trials seen in Table 1 (maximum, $<$10-fold) may have been due to differences in batches of primary chicken kidney cells. These variations were particularly troublesome when the pH sensitivity of a virus strain bordered on acid stability-lability (e.g., JFB9 and 10). Perhaps the use of a common cell source, such as a pool of primary chicken kidney cells (stored with the addition of a protectant at -196 C), would help reduce this test variability. The storage of cells in the frozen state will need investigating, however, to determine whether the loss in sensitivity (titer reductions of 100-fold or more) resulting from the use of dimethyl sulfoxide-frozen cells (Cowen, unpublished data) is due to dimethyl sulfoxide, freezing, or both. Perhaps glycerin or polyvinylpyr-

TABLE 1. *pH stability tests on IBV strains*

Virus	Cell culture passage	Titer (log_{10} PFU/ml)					
		Trial 1			Trial 2		
		pH 7.2	pH 3.0	Titer reduction	pH 7.2	pH 3.0	Titer reduction
Massachusetts 41	24	7.0	≤2.0	≥5.0	6.1	≤2.0	≥4.1
Connecticut	23	6.2	4.5	1.7	6.1	5.1	1.0
Clark 333	32	6.5	5.5	1.0	6.8	5.3	1.5
Holte	22	6.8	≤2.0	≥4.8	6.2	≤2.0	≥4.2
Gray	20	5.5	4.6	0.9	5.8	5.2	0.6
JMK	19	6.8	4.4	2.4	6.1	4.3	1.8
Iowa 33	18	7.3	6.0	1.3	7.4	6.2	1.2
Iowa 97	15	6.9	5.2	1.7	6.3	5.1	1.2
Iowa 609	16	7.4	6.0	1.4	7.1	6.1	1.0
SE-17	18	6.8	3.8	3.0	5.6	3.3	2.3
JFB 2	11	6.0	4.8	1.2	6.6	5.6	1.0
JFB 5	11	6.0	4.8	1.2	6.4	5.3	1.1
JFB 8	11	6.4	5.4	1.0	7.8	6.8	1.0
JFB 9	16	6.0	5.1	0.9	6.7	5.3	1.4
JFB 10	11	6.2	5.4	0.8	6.8	5.8	1.0
JFB 27	14	5.7	4.6	1.1	6.7	5.4	1.3
JFB 28	12	6.4	5.4	1.0	6.8	5.7	1.1
CELO[a]	2	7.0	6.9	0.1	7.5	7.3	0.2
ILT[b]		5.2	2.2	3.0	5.4	2.8	2.6

[a] Phelps strain, acid-stable virus control.
[b] Infectious laryngotracheitis virus, acid-labile virus control.

rolidone could be used as protectants if dimethyl sulfoxide is incriminated. Trial variations were even more pronounced (Table 2, maximum of >100-fold) when infectivity end points were assayed as mean tissue culture infective doses. If, however, circumstances dictate the use of a 50% end point assay, then we would recommend increasing (from three to five or more) the number of replicates per dilution. Variability of the magnitude seen during this investigation, when using the 50% end point assay, is unacceptable and points out the need for uniform test procedures between laboratories in order to compare results. As a result of this investigation, we conclude that pH stability is a variable feature of the avian coronaviruses. Further, we encourage other investigators working with IBV or other coronaviruses (of various species origin) to test their virus strains by using a procedure similar to that described herein, so as to facilitate valid comparisons. Once a method has been adopted and extensively tested, perhaps we will find pH stability to be the basis for an additional subdivision of coronaviruses. Such a phenomenon is not unique among virus families (i.e., picornaviruses) (17).

TABLE 2. *pH stability tests on IBV strains; trial variability seen when calculating 50% infectivity end points*

Virus	Cell culture passage	Trial	Titer (log_{10} $TCID_{50}$/ml)[a]		
			pH 7.2	pH 3.0	Titer reduction
Connecticut	23	1	6.5	5.8	0.7
		2	6.5	5.3	1.2
		3	6.5	3.8	2.7
Gray	20	1	6.5	5.3	1.2
		2	6.3	5.5	0.8
		3	6.3	5.8	0.5
JFB 28	12	1	6.8	5.5	1.3
		2	7.3	3.8	3.5

[a] $TCID_{50}$, mean tissue culture infective dose.

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